Expression Analysis of Genes Responsible for Rosmarinic acid Biosynthesis and HPLC Quantification Method Development and Validation of Rosmarinic acid from *Isodon rugosis*

Saira khan (sairaasifkhan@yahoo.com)
University of Haripur, Pakistan
Anum Imtiaz
COMSATS University Islamabad, Abbottabad Campus, Pakistan
Clauvis Nji Tizi Taning
Ghent University, Belgium
Ihsan Ul Haq
National agriculture research Center, Islamabad, Pakistan
Guy Smagghe
Ghent University, Belgium
Mohammad Maroof Shah
COMSATS University Islamabad, Abbottabad Campus, Pakistan

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Abstract

Natural products from plants, either as pure compounds or as standardized extracts, offer unlimited prospects for new pesticide discovery. In screening programs, because of increased chemical diversity demand, in search of pesticides from natural products, interest mainly in harmless plants has developed all over the world. Botanicals comprise of several types of bioactive compounds. In our previous publications, bioactive pesticidal compound; rosmarinic acid (RA) was isolated from the plant, *Isodon rugosus* and was identified by using various analytical techniques. In this study two key genes, hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS), known to involve in biosynthesis of RA were targeted to clone from *Isodon rugosus*. Only one of these genes, HPPR was successfully cloned in *I. rugosus* and its cDNA was fully sequenced through RACE (Rapid Amplified cDNA ends) PCR, which consequently will open the way to explore all other genes responsible for biosynthesis of rosmarinic acid. The expression of HPPR was analyzed in different parts of plant and it was found that RA was expressed in all parts of the plant. Further, RA quantification was performed on RP-HPLC using C18 column, giving a maximum absorbance at 310 nm in isocratic conditions. The methodology was found selective and robust to quantify 1.60+0.14gm/kg RA in *I. rugosus* with sensitivity of LOD 1.32 µg/ml, and LOQ 4.41 µg/ml. The molecular knowledge regarding biosynthetic pathway and significant quantity of RA in this plant will help in biotechnological production of RA and to produce insect resistant plants through genetic engineering approaches.

Introduction

Due to mounting hazards and cost of synthetic pesticides, natural products derived from plants provide the hope for sustainable pest management with reduced environmental and health hazards in the future. Plants produce number of secondary metabolites due to their contact with insects and these metabolites can act in many different ways against insects (Akhtar and Isman 2004; Pavela, 2008; Basukriadi and Wilkins, 2014). As, plants give additive or synergistic effects of many strong and weak insecticidal activities, the purification and structure determination of the active principle is necessary to improve bioefficacy of active principles (Khan et al., 2018). Koul and Walia (2009) stated that plant based pesticides and their active principle compounds are the safer approach. Due to stress condition defense mechanism of plants become activated, results in the development of many biochemical signals, which leads towards the induce expression of genes of biosynthetic pathway of secondary metabolite. The discovery of active compounds leads towards the possibility to manipulate their biosynthetic pathways, increasing their yield through cell and tissue culture (Kim et al. 2009).

Isodon rugosus (Wall. ex Benth.) Codd is a medicinal plant mostly found in the northern of Pakistan (Khan et al., 2007; Janbaz et al., 2014). This plant is rich in bioactive compounds that have profound applications in pharmaceutical industries and agriculture (Khan et al., 2016, 2017, 2018). As this plant is aromatic, its essential oil composition is examined through GC-MS in many studies (Janbaz et al 2014; Zeb et al., 2014, 2017). In this plant, the occurrence of pentacyclic triterpenes and caffeic acid phenolic derivatives have been also described (Duan et al., 2017). Many phytochemicals with significant importance have also been reported in this plant including rosmarinic acid (RA), caffeic acid, pentacyclic triterpenoids (plectranthoic acid (PA), oleanolic acid (OA), betunilic acid (BA)),and other phenolic compounds (Batista et al., 1994; Abdel-Mogib et al., 2002, Shetty and Wahlqvist 2014; Duan et al., 2017).

Rosmarinic acid (RA) is a phenolic compound showing many potential bioactivities; insecticidal, antibacterial, antifungal, astringent, anti-oxidative antimutagen, antiviral and anti-inflammatory (Dashti et al., 2016; Fernando et al., 2016; Han et al., 2015; Lucarini, et al., 2013; Trocsanyi et al., 2015; Yerra et al., 2014). Many plants has been screened for RA (Iswandana et al., 2016; Benedec et al., 2015; Petersen, 2013) and for RA production through biotechnology and metabolic engineering has been studied in many reports (Wu et al., 2016; Lu et al., 2013; Kim et al 2014; Kim et al., 2014; Khojasteh et al., 2014; Ellis et al., 1970). But in recent years due to increasing demand for RA plant sources become insufficient.

It is reported that two amino acids, L-phenylalanine and L-tyrosine, are precursor of RA in a biosynthetic pathway of *Coleus blumei*. In the biosynthesis, eight genes have been involved as described in *Coleus blumei* by many researchers (Petersen et al., 1993, 1994; Kim et al., 2004; Eberle et al., 2009). Two key genes out of total eight genes were targeted for cloning from *Isodon rugosus*. These two targeted key genes were rosmarinic acid synthase gene (RAS) and hydroxyphenylpyruvate reductase (HPPR) gene. (Fig. 1)

Regarding biosynthetic pathway, RA genes have been cloned and characterized from many plants including *C. blumei* (Kim et al., 2004), *Agastache rugosa* (Pham et al., 2012), *Perilla frutescens* (Lu et al, 2013), *Melissa officinalis* (Weitzel and Petersen, 2011), *Salvia miltiorrhiza* (Xiao et al., 2011; Song et al., 2012) and *Dracocephalum tanguticum* (Li et al., 2017). As biosynthetic genes of RA are commonly shared by biosynthesis of various secondary metabolites, interpretation of the potential pathway in case of RA biosynthesis would assist the essential molecular manipulation to increase the RA quantity in *Isodon rugosus*.

Materials and method

1. Expression analysis of genes responsible for the biosynthesis of isolated pesticidal compound; rosmarinic acid

1.1 Plant material

Leaves of the plant, *Isodon rugosus* were collected in liquid nitrogen and brought to the laboratory where they were washed with RNAase free water and after drying they were kept at -80°C in laboratory for 2 h. After freezing at -80°C, leaves were immediately transferred to sterilized zipper bags and were placed in lyophilizer where they were freeze dried for 72 h.

1.2 RNA extraction and cDNA synthesis

RNA from leaves of *I. rugosus* was extracted by using commercial kit (Qiagen) by following a standard protocol. For RNA extraction, 40 mg of freeze dried leaves were taken. The extracted RNA was immediately stored at -80°C in a freezer. Quality of extracted RNA was checked by running 2 µL of RNA on a gel in 1.5 X TE buffer at 100 volts for 25 to 30 min and gel was observed. The RNA quantity was checked by Nanodrop DeNovix DS-11 Spectrophotometer, by using RNAase free water as a blank and 260/230 and 260/280 absorbance ratios were also analyzed. cDNA was synthesized from 1 µg of RNA using SuperScript™ II Reverse Transcriptase by Thermo Fisher Scientific**.** DNA quantity was checked by Nanodrop DeNovix DS- 11 Spectrophotometer, by using nuclease free water as a blank and 260/230 and 260/280 absorbance ratios were also observed.

1.3 Target genes

In the biosynthetic pathway of rosmarinic acid, two key genes out of total eight genes were targeted for cloning from *Isodon rugosus*. These two targeted key genes were rosmarinic acid synthase gene (RAS) and hydroxyphenylpyruvate reductase (HPPR) gene. (Fig 1)

1.3.1 PCR amplification of rosmarinic acid synthase gene (RAS)

Three sets of primers were used for amplification of rosmarinic acid synthase gene, their names, sequence and the plant species from which these primes were designed are listed in the table 1. Degenerate primers were designed by choosing highly conserved region after aligning sequences for RAS from closely related plant species, *Melissa officinalis*, *Perilla frutescens*, *Salvia miltiorrhiza* and *Solenostemon scutellarioides* obtained from NCBI Gene Bank (http://www.ncbi.nlm.nih.gov). Gradient PCR amplification was carried out for 33 cycles in 20 µL reaction mixture. This mixture contained 0.9 µL cDNA, 0.2 µL Taq DNA polymerase, 0.6 µL 10mM dNTPs, 0.6 µL each 10 µM forward and reverse primers, 0.6 µL 50 mM $MgCl_2$ and 2 µL 10X PCR buffer. The conditions used for amplification through gradient PCR were; denaturation at 94°C for 2 min, then 33 cycles starting with denaturation at 94°C for 30 sec, annealing temperatures used were 10 temperatures from 50 to 60°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min.

Table 1: Primers used in the amplification of rosmarinic acid synthase gene

Primers	Sequence	Plant specie
F-RAS1	5'-ATTACATATGAAGATAGAAGTCAAAGA CTC-3'	*Coleus blumei*
R-RAS1	5'-TAGGATCCTCATCAAATCTCATAAAACA ACTTCTC-3'	
F-RAS2	5'-AAGGGAATTTCCACGTACCC-3'	*Melissa officinalis*
R-RAS2	5'-ACCCAGCTAATCACCCACAA-3'	
F-RAS3	5'-GACGAAGCTCCACATCCCCTTC-3'	*Melissa officinalis*
R-RAS3	5'-GCGCTCCATATGCTGCGTGT- 3'	

1.3.2 PCR amplification of hydroxyphenylpyruvate reductase (HPPR)

One set of primers was used for amplification of HPPR gene from *I. rugosus*. Primer's names, sequence and the plant species from which these primes were designed are listed in the table 2. Degenerate primers were designed by choosing highly conserved region after aligning sequences for HPPR from closely related plant species, *Perilla frutescens*, *Solenostemon scutellarioides*, *Salvia officinalis* and *Salvia miltiorrhiza* obtained from NCBI Gene Bank (http://www.ncbi.nlm.nih.gov). PCR amplification was carried out in 20 µL reaction mixture. This mixture contained 0.9 µL cDNA, 0.2 µL Taq DNA polymerase, 0.6 µL 10mM dNTPs, 0.6 µL each 10 µM forward and reverse primers, 0.6 µL 50 mM $MgCl_2$ and 2 µL 10X PCR buffer. The conditions used for amplification in PCR were denaturation at 94°C for 2 min, 33 cycles started with denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min.

Table 2: Primers used in amplification of hydroxyphenylpyruvate reductase gene

Primers	Sequence	Plant Specie
F-HPPR	5'- GCGCTGCCGAAATTGGAGAT- 3'	*Perilla frutescens*
R-HPPR	5'-CGTTTCTGGAGTCAGCGCACA-3'	

PCR products were analyzed on gel electrophoresis in 1.5 X TE buffer at 100 volts for 30 to 35 min and then observed and photographed by Bio-Rad's gel documentation system. The amplified bands were cut from the gels and after purification with Wizard® SV Gel and PCR Clean-Up System and sequenced.

1.4 3' and 5'RACE PCR for sHPPR (HPPR from *I. rugosus*)

After getting the sequence for conserved region of sHPPR from *I. rugosus*, rapid amplification of cDNA ends (RACE) PCR was performed to get full length sequence for sHPPR from *I. rugosus*. For 3' and 5' RACE PCR SMARTer™ RACE cDNA amplification Kit from Clontech was used. All the reagents and chemicals

were provided by the kit except RNA that was isolated from *Isodon rugosus* in the laboratory. After PCR, both synthesized 3' and 5' RACE cDNAs were kept at -20°C until used.

1.4.1 3' and 5'RACE PCR

For 3' and 5'RACE PCR, 50 µL reaction was prepared. For each 50 µL reaction, 34.5 µL of PCR-Grade water, 5 µL of 10X Advantage 2 PCR Buffer, 1 µL 10 mM dNTP Mix and 1 µL of 50X Advantage 2 Polymerase Mix were mixed in ependorf tubes on ice to prepare the master mix. After mixing and spinning, for 3'RACE PCR amplification, 2.5 µL of 3' RACE synthesized cDNA, 5 µL of 10X UPM (universal primer mix), 1 µL of 10 µM 3'sHPPR gene specific primer (Table 3) and 41.5 µL of master mix were added in PCR tubes on ice. For 5'RACE PCR amplification, 2.5 µL of 5'RACE synthesized cDNA, 5 µL of 10X UPM (universal primer mix), 1 µL of 10 µM 5'sHPPR gene specific primer and 41.5 µL of master mix were added in PCR tubes on ice. After spinning, each PCR tube was incubated under the following conditions, 5 cycles of 94°C for 30 sec and 72°C for 10 min, 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 10 min, and finally 25 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 10 min.

Table 3: sHPPR gene specific primers for 3' and 5'RACE PCR amplification

Primers	Sequence
3'RACE sHPPR	5'-GATTTGGCGATCGGGTTGATGTTG-3'
5'RACE sHPPR	5'-CCCAATTCTGCCCAATCCTATGATGC-3'

Both 3' and 5'RACE PCR products were run in 10X TE buffer gel electrophoresis. After running and after washing in distilled water gels were then observed and photographed by Bio-Rad's gel documentation system. The amplified bands were cut from the gel and after purification with Wizard® SV Gel and PCR Clean-Up System and were sequenced.

1.5 Expression of HPPR in leaves, roots and stems of *I. rugosus*

1.5.1 RNA extraction and cDNA synthesis

Lyophilized leaves, roots and stems were used for the extraction of RNA. RNA was extracted three times on three different days from leaves; roots and stems in order to accomplished three biological repetitions. Qiagen kit was used to extract RNA from all samples. Extracted RNA was analyzed through gel electrophoresis before DNAase treatment. For DNAase treatment of the extracted RNA, TURBO DNA-Free^{TM} Kit by Thermo fisher Scientific was used. Extracted RNA was collected carefully and kept at -80°C. After DNAase treatment each RNA sample was analyzed through gel electrophoresis before cDNA synthesis. cDNA was synthesized from 1 µg of each RNA using SuperScript^{TM} II Reverse Transcriptase by Thermo Fisher Scientific. The synthesized cDNA was store at -20°C. RNA and DNA quantity was checked by Nanodrop DeNovix DS- 11 Spectrophotometer, by using nuclease free water as a blank and 260/230 and 260/280 absorbance ratios were also observed.

1.5.2 PCR amplification

For PCR amplification 20 µL of reaction mixture was used for each reaction of leaves, roots and stems, separately. For each reaction, 0.9 µL cDNA, 0.2 µL Taq DNA polymerase, 0.6 µL 10mM dNTPs, 0.6 µL each 10 µM forward and reverse primers for HPPR and for actin gene respectively, 0.6 µL 50 mM $MgCl_2$ and 2 µL 10X PCR buffer were added in PCR tubes. Actin gene was used as reference gene in each leaves, roots and stem reaction. Actin and HPPR gene primers used in the reactions are listed in the table 4. The conditions used for amplification in PCR were denaturation at 94°C for 2 min, 33 cycles of denaturation at 94°C for 30 sec, primer annealing at 56°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min.

Table 4: Actin and HPPR primers used in PCR amplification of HPPR and actin gene from roots, leaves and stem of *Isodon rugosus*

Primers	Sequence	Plant Specie
F-Actin	5'-GAGGTTGGATCTTGCTGGTC-3'	*Melissa officinalis*
R-Actin	5'-GCTCGGCAGTTGTGGTAA-3'	
F-HPPR	5'-GCGCTGCCGAAATTGGAGAT-3'	*Perilla frutescens*
R-HPPR	5'-CGTTTCTGGAGTCAGCGCACA-3'	

2. Quantification of rosmarinic acid in *Isodon rugosus*

2.1 Chemicals

Rosmarinic acid (RA) standard with 98% purity (Sigma-Aldrich® Co., Germany), HPLC grade acetonitrile, methanol, anhydrous formic acid (Merk, Germany), and Millipore deionized water were used in this study. All other reagent grade chemicals utilized in HPLC analysis were used without further purification.

2.2 Plant material

The crude methanolic extract of *I. rugosus* plant extracted in the lab and analyzed in the previous studies (Khan et al., 2016, 2017, 2018) was used in the current study.

2.3 Instruments

Perkin Elmer HPLC System comprised of Binary LC-250 pump, UV/VIS LC-295 detector, PE Nelson 900 Series Interface, TurboChrom Chromatographic software, reversed-Phase C18 column with dimensions of 5 µm Spherical Silica, 4.6 mm x 250 mm column, analytical balance (Sartorius), pH meter (Thermo Orion 410), Ultrasonic bath (Elma, Switzerland), Millipore filtration assembly with vacuum pump.

2.4 Chromatographic conditions

Column: C-18, 250mm X 4.6mm, 5µ

Flow rate: 1.0 ml /min

Column temperature: Ambient

Injection volume: 20 µl

Run time: 15 minutes

Elution: Isocratic

Wavelength: 310nm

2.4.1 Preparation of mobile phase

The Mobile Phase composed of deionized water containing acetic acid (0.1 % v/v, pH adjusted to 3.3 ±0.1) and acetonitrile in the ratio of 70:30 (v/v), was filtered and degassed before use.

2.4.2 Standard preparation

The standard solutions were prepared in methanol. Stock solution of 500 µg/ml was prepared by dissolving 5 mg of crystalline rosmarinic acid in 10 mL of methanol. Further working dilutions of 100, 50, 25, 12.5, 6.25 and 3.12 µg/mL were prepared.

2.4.3 Sample preparation

A total of 123 mg of methanolic extract of *I. rugosus* was sonicated with 10 mL methanol for 30 min and transferred in sealed volumetric flasks at room temperature. The sample was filtered from 0.2µm syringe membranes. The 2.5 mL of the filtrate was directly diluted in 2.5 mL of methanol and subsequently six dilutions were made.

2.4.4 Quantification Procedure

Quantification procedure was started by injecting 20µl of blank solution and standard solution to rule out any contamination or interference of the solvent compound on the RA concentration in the sample.

2.4.5 System suitability test

System suitability was evaluated before and during the chromatographic runs, by following the Food and Drug Administration (FDA) guidelines (US Food and Drug Administration 2013; Sangita et al., 2013). To ensure the performance of the system, all the chromatographic parameters listed below were determined.

- Theoretical plate (N) value to check the separation efficiency of the column
- System precision in terms of retention time (RT)
- Tailing factor (T) value to check the quality and sharpness of the peak
- Peak area repeatability (expressed as Relative Standard Deviation, R.S.D; n = 6) RSD≤1% for N≥5 is desirable
- Linearity response was assured by constructing calibration curves and regression line analysis for RA standard by using the Eq. [1]

$y = ax + b$ ———————————— [1]

2.4.6 Method validation

Reliability of the analytical method framed during this study for quantification of RA in *I. rugosus* was validated in terms of linearity, selectivity, sensitivity, repeatability, precision, and robustness as per single laboratory validation protocol (Kollipara et al., 2011; Sangita et al., 2013).

Linearity response for the method was also determined by injecting triplicates of the samples of *I. rugosus* at six different concentrations into the LC apparatus on 3 different days. To assess the linearity of method calibration curves using the peak areas of RA versus concentrations of samples were constructed and regression analysis was performed by using the Eq. [1].

To evaluate the precision and to evaluate the between-day and between-week precision by calculating R.S.D. of the response obtained, six concentrations of herbal material were evaluated in triplicate on two consecutive days of two weeks.

To find out any interference by other compounds in the dilutions, the method selection was performed by calculating the average retention time of RA standard and herbal material at six different concentrations.

Sensitivity of the method for RA was predicted by LOD and LOQ values, calculated by means of Eqs. [2] and [3] respectively where bsd is the standard deviation of the regression line intercept.

LOD = 3 (bsd) / a ----------[2]

LQO = 10 (bsd) / a---------- [3]

The robustness of test method was confirmed by carrying out mobile phase variation i.e. (ratio of acetonitrile alteration between 29-30%), flow rate variation ±10% i.e. (1.1mL - 1.3mL/min) and changing the chemical brand of acetonitrile from Merck to BDH.

Results

1. Expression analysis of genes responsible for the biosynthesis of isolated pesticidal compound; rosmarinic acid

In the biosynthesis of rosmarinic acid, eight genes have been involved as described in *Coleus blumei* by many researchers (Petersen et al., 1993, 1994; Kim et al., 2004; Eberle et al., 2009). In this study, two keys genes; rosmarinic acid synthase gene (RAS) and hydroxyphenylpyruvate reductase gene (HPPR) of this biosynthetic pathway were targeted for cloning from *I. rugosus*.

1.1. PCR amplification of rosmarinic acid synthase gene (RAS)

Three sets of primers were used for amplification of rosmarinic acid synthase gene from *I. rugosus*. Gradient PCR was performed by using 10 different primer annealing temperatures between 50°C to 60°C under specified conditions.

1.1.1. Amplification with first set of primers

With first set of primers, F-RAS15'-ATTACATATGAAGATAGAAGTCAAAGACTC-3'and F-RAS1 5'-TAGGATCCTCATCAAATCTCATAAAACAACTTCTC-3', there was no amplification observed for rosmarinic acid synthase gene after analyzing gel by Bio-Rad's gel documentation system.

1.1.2. Amplification with second set of primers

Amplification with second set of primers, F-RAS2 5'-AAGGGAATTTCCACGTACCC-3' and R-RAS2 5'-ACCCAGCTAATCACCCACAA-3',through gradient PCR from 50°C to 60°C primer annealing temperature resulted in multiple bands on gel after observing by Bio-Rad's gel documentation system. Expected size of the band was 786 bp and this band was sequenced after purification through Wizard® SV Gel and PCR Clean-Up System (Promega). There was no sequence observed for RAS after doing BLAST for the obtained sequence through NCBI Gene bank (Figs. 2 and 3).

1.1.3. Amplification with third set of primers

Amplification with third set of primers, F-RAS35'-GACGAAGCTCCACATCCCCTTC-3'and R-RAS3 5'-GCGCTCCATATGCTGCGTGT-3', through gradient PCR using 50°C to 60°C annealing temperatures results in multiple bands on gel after observing by Bio-Rad's gel documentation system. All the bands from the gel were purified separately through Wizard® SV Gel and PCR Clean-Up System (Promega), and were sequenced. There was no sequence observed for RAS in case of all purified bands (Fig. 4).

1.2. PCR amplification of hydroxyphenylpyruvate reductase (HPPR)

Amplification of HPPR was performed by using a set of primers, F-HPPR 5'-GCGCTGCCGAAATTGGAGAT-3' and R-HPPR 5'-CGTTTCTGGAGTCAGCGCACA-3' at 56°C primer annealing temperature. PCR amplification was observed at 438 bp after analyzing the gel by Bio-Rad's gel documentation system. This band was purified with Wizard® SV Gel and PCR Clean-Up System and sequenced. It was confirmed from the obtained sequence that the amplified product was sequence of HPPR from *I. rugosus* (sHPPR) (Fig. 5).

1.2.1. sHPPR sequence (HPPR from *I. rugosus*)

>sHPPR

GGTCGACTTAATCAAGTGTAAGGAGAAGGGGATTAGGGTTACCAACACGCCAGATGTGCTGACCGA
TGACGTCGCGGATTTGGCGATCGGGTTGATGTTGGCGGTTTTGAGGCGGATTTGTGAGTGTGATAAGTATGTG
AGGAGGGGGGCGTGGAAACTTGGTGACTTCAAGTTGACGACTAAGTTCAGTGGCAAAAGAGTTGGCATCATAGGATTGGGCAGAATTGGGTTAGCAATTGCTGAGCGC
TACTCAAGATCCGAGAAAACCTACACGAACTACAAGTACTATGACAGCGTTGTTGAGTTGG
CATCAAACAGTGACATCTTAGTGGTAGCATGTGCGCTGACTCCAGAAACG

1.2.2. sHPPR phylogenetic tree

NCBI blast analysis and alignment in Clustal omega of sHPPR and HPPR in other plant species confirmed the identity of sHPPR as HPPR. A phylogenetic tree was also generated to show the relatedness of sHPPR to HPPR in other plant species (Fig. 6).

1.2.3. Alignment of amplified sHPPR with the conserved region amplified from other reported plant species

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CLUSTAL O (1.2.1) multiple sequence alignment


I. rugosus       ----------------------------------GGTCGACTTAATCAAGTGTAAGGAG
P. frutescens    GAGATAGTTTCGTGCTTCAGCGTGGGATTGGATAAGGTCGACTTGATCAAGTGCAAGGAG
S.scutellarioides GAGATTGTGTCGAGCTTTAGCGTGGGTCTGGATAAGGTTGACTTGATCAAGTGTGAGGAG
S. officinalis   GAGATAGTTTCGAACTTTAGCGTGGGATTGGACAAAGTCGACTTGGTCAAGTGCAAGGAG
S. miltiorrhiza  GAGATAGTTTCGAGCTTCAGCGTGGGATTGGACAGAATCGACTTGCTCAAGTGTAAGGAA
                                                  * *****  *******  ****

sHPPR            AAGGGGATTAGGGTTACCAACACGCCAGATGTGCTGACCGATGACGTCGCGGATTTGGCG
P. frutescens    AAGGGGATTAGGGTTAGCAACACGCCTGATGTGCTCACCGATGACGTGGCGGATTTGGCG
S. scutellarioidesAAGGGGGTTAGGGTTACCAACACGCCCGATGTGCTGACGGATGACGTCGCGGATTTGGCG
S. Officinalis   AAGGGGGTTAGGGTTACCAACACGCCCGATGTGCTGACGGATGACGTAGCGGATTTGGCG
S. miltiorrhiza  AAGGGGATTAGGGTTACCAACACGCCCGATGTGCTGACGGAAGACGTCGCGGATTTGGCG
                 ****** ********** ********** ********* ** ** ***** ************

sHPPR            ATCGGGTTGATGTTGGCGGTTTTGAGGCGGATTTGTGAGTGTGATAAGTATGTGAGGAGG
P. frutescens    ATCGGGTTGATGTTGGCGGTTTTGAGGCGCATTTGTGAGTGTGATAAGTATGTGAGGAGA
S. scutellarioidesATCGGGTTGATTTTGGCGGTTTTGAGGCGGATTTGTGAGTGTGATAAGTATGTGAGGAGA
S. Officinalis   ATCGGGTTGATATTGGCGGTTCTCAGGCGGATTTGTGAGTGCGATAAGTATGTCAGGAGA
S. miltiorrhiza  ATCGGGTTGATGCTGGCGGTTCTGAGGCGGATTTGCGAGTGCGACAAGTATGTGAGGAGC
                 ***********  ********* * ***** ***** ***** ** ********* *****

sHPPR            GGGGCGTGGAAACTTGGTGACTTCAAGTTGACGACTAAGTTCAGTGGCAAAAGAGTTGGC
P. frutescens    GGGGCGTGGAAATTTGGCGACTTCAAGTTGACTACTAAGTTCAGCGGCAAAAGAATTGGT
S. scutellarioidesGGGGCGTGGAAATTTGGAGACTTCAAGTTGACGACTAAGTTCAGCGGTAAAAGAGTTGGC
S. officinalis   GGGGCGTGGAAATTGGGAGACTTCAAGTTGACTACTAAGTTCAGCGGCAAAAGAGTTGGC
S. miltiorrhiza  GGGGCGTGGAAATTAGGAGACTTCAAGTTGACTACTAAGTTCAGTGGCAAAAGAGTTGGC
                 ************ * ** ***************** *********** ** ****** ****

sHPPR            ATCATAGGATTGGGCAGAATTGGGTTAGCAATTGCTGAGCGCGCAGATGCATTTGATTGT
P. frutescens    ATCATAGGATTGGGCAGAATTGGCTTAGCAGTTGCTGAGCGAGCAGAGGCATTTGATTGC
S. scutellarioidesATCATAGGATTGGGCAGAATCGGGTTAGCAGTTGCTGAGCGAGCAGAGGCATTTGATTGT
S. officinalis   ATCATAGGATTGGGCAGAATCGGGTTAGCAGTTGCTGAGCGAGCAGAGGCATTTGATTGC
S. miltiorrhiza  ATCATAGGATTGGGCAGAATCGGGTTAGCAGTTGCTGAGCGAGCAGAGGCATTCGATTGT
                 ********************** ** ****** ********** ***** ***** *****

sHPPR            CCCATCAGTTACTACTCAAGATCCGAGAAAACCTACACGAACTACAAGTACTATGACAGC
P. frutescens    CCCATCAACTACTACTCAAGATCCAAGAAACCCAACACAAACTACACCTACTATAGCAGC
S. scutellarioidesCCAATCAGTTACTTTTCAAGATCCAAGAAACCCAACACAAACTACACATACTATGGCAGC
S. officinalis   CCCATCAATTACTACTCAAGATCCAAGAAAGCCAACACAAACTACACATACTATGGCAGC
S. miltiorrhiza  CCCATCAATTACTACTCAAGATCCAAGAAAGCCAACACAAACTACACGTACTATGGCAGC
                 ** ****  ****  *********** ***** ** **** ********  ******  ****

sHPPR            GTTGTTGAGTTGGCATCAAACAGTGACATCTTAGTGGTAGCATGTGCGCTGACTCCAGAA
P. frutescens    GTTGTTGAATTGGCATCAAACAGTGACATTCTAGTCGTAGCATGTGCGCTGACTCCAGAA
S. scutellarioidesGTTGTAGAATTGGCGTCAAACAGTGACATCCTAGTCGTAGCATGTCCCCTGACTCCAGAA
S. officinalis   ATTGTTGAATTGGCATCCAACAGTGACATCCTAGTGGTAGCATGTGCGCTGACTCCAGAA
S. miltiorrhiza  GTTGTTGAATTGGCATCAAACAGCGATATCCTAGTGGTAGCATGTGCCCTGACTCCAGAA
                  **** ** ***** ** ***** ** **  **** ********* * ************

sHPPR            ACG
P. frutescens    ACG
S. scutellarioidesACA
S. officinalis   ACA
S. miltiorrhiza  ACA
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1.3. 3' and 5' RACE PCR for sHPPR

For full length amplification of sHPPR, RACE PCR was performed. After PCR, PCR samples were run on gel in 1.5X TE buffer for 30 min. After the gel electrophoresis the gels were observed by Bio-Rad's gel documentation system. The both 3' and 5'PCR products were purified from the gels with Wizard® SV Gel and PCR Clean-Up System and were sequenced (Fig. 7).

1.3.1. 3'RACE PCR product sequence for sHPPR

>3'RACE

AAGTATGTGAGGAGGGGGGCGTGGAAACTTGGTGNNTTCAAGTTGACGACTAAGTTCAGTGGC
AAAAGAGTTGGCATCATAGGATTGGGCAGAATTGGGTTAGCAATTGCTGAGCGCGCAGATGCAT
TTGATTGTCCCATCAGTTACTACTCAAGATCCGAGAAAACCTACACGAACTACAAGTACTATGAC
AGCGTTGTTGAGTTGGCATCAAACAGTGACATCTTAGTGGTAGCATGTGCCCTGACTCC

1.3.2. 5'RACE PCR product sequence for sHPPR

>5'RACE

CTGAACTTAGTCGTCAACTTGAAGTCACCAAGTTTCCACGCCCCCCTCCTCACATA
CTTATCACACTCACAAATCCGCCTCAAAACCGCCAACATCAACCCGATCGCCAAATCCG
CGACGTCATCGGTCAGCACATCTGGCGTGTTGGTAACCCTAATCCCCTTCTCCTTACAC
TTGATTAAGTCGACCTTATCCAGACCTACGCTATAGCTCGAAACTATCTCCAATTTCGGCAG
TGCATCGATCAGCTCGGCGTCGGCGCCGGCGGTGGCGTTTCCGACCACCGCGCGGATGGA

CTCGGCCTGCCGAGTGAGAAACTCGCGCTGCGCCGGCTGAGCCCAGTAACGGAAGAGCT
TGAACCGCTTGTCGAGCTCTTGCTCCAAGTAGTTGCTCATCGGGCACAACATCAGAACACC
GATCGCCTCCATTTTAACTGCAGCAGCGGCGTCGGCGTCGGCGGCGGTGGCGGGGGATGG
AGATGGTGTTTGAGTGGTAGTTGAGA

1.4 Expression of HPPR in leaves, roots and stems of I. rugosus

HPPR gene was amplified from, roots, leaves and stems of *I. rugosus* to confirm its presence in these parts of the plant through PCR at 56°C primers annealing temperature. Actin gene was also amplified from these parts of the plant, used as a control. RNA from all these parts was extracted on three different days and used for this analysis. Amplification of HPPR gene in leaves, roots and stems has confirmed its expression in these parts of the plant, *I. rugosus* (Fig. 8).

2. Quantification of rosmarinic acid in Isodon rugosus

2.1. System suitability test

System suitability tests are performed to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be performed. The system suitability test of RA (Table 5) indicates that all the system performance parameters are in accordance with the literature specifications (US Food and Drug Administration 2013; Sangita et al., 2013).

Table 5
System suitability RA, rosmarinic acid; N, theoretical plates; T, tailing factor; RSD, Relative Standard Deviation; RT, retention time

System suitability parameters	N	T	Repeatability (RSD%; n = 6)	RT (RSD%; n = 6)
RA Standard	2442.02	1.421	0.8148%	0.7499%
I. Rugosus Extract	2428.41	1.702	0.9266%	1.4936%
Literature specifications	> 2000	≤ 2.0	< 1.0%	< 2%

Calibration curves in (Fig. 9) showed a linear response of the system within the range of 3.12–100.0 ppm over a 3-day validation period. Correlation coefficients (R^2) of 0.9988, 0.9957 and 0.9984 were obtained, respectively, in the first, second and third day of analysis. According to ANVISA specifications value of $R^2 \geq 0.99$ are acceptable. The equations of each calibration curve in the regression analysis for the first, second and third day were Eqs. [5], [6], and [7], respectively (Fig. 10).

y = 66788x − 107820 [5]

y = 66503x -56325 [6]

y = 67205x − 112347 [7]

2.2. Method validation

During selectivity test interference of no other compound was found with the retention time of RA standard at six different concentrations (6.50 ± 0.04) and in the herbal material (6.33 ± 0.07). Figures 11 and 12 show the chromatographic profile of methanol, RA standard and *I, rugosus* plant extract respectively.

To check the linearity of the method calibration curves for *I. rugosus* plant extract samples for 6 concentrations were calculated over a 3 day validation period (Fig. 13). This analytical method for RA quantification in *I. rugosus* presented a suitable linearity (Fig. 14). Correlation coefficients (R^2) for extract were 0.9899, 0.9909 and 0.9904 calculated for the regression line analysis for the first, second and third day were Eqs. [8], [9], and [10], respectively and were found acceptable according to ANVISA specifications value of $R^2 \geq 0.99$.

y = 198848x + 338539 [8]

y = 200500x + 322573[9]

y = 214855x + 191009[10]

Sensitivity of the analytical method was assessed by means of the LOD and LOQ determinations. LOD is the minimum concentration of the analyt in analysis that can be detected with satisfactory level of consistency and LOQ is the minimum concentration value that can be quantified precisely and accurately by the analytical method in the sample (Kollipara et al., 2011). LOD and LOQ values were 1.32 µg/mL and 4.41 µg/mL, respectively, which means that the current method has sensitivity to detect the rosmarinic acid in concentrations greater than 1.32 µg/mL, and quantify up to 4.41 µg/mL with adequate precision and accuracy in concentrations. In previous methods, LOD and LOQ values of 0.708 and 2.361 µg/mL, and LOD and LOQ values of 7.24 and 24.13 ng/mL are reported for RA quantification in Rosemary (Wang et al., 2004; Couto et al., 2011).

Precision test, another very important parameter for the validation of method has been performed. The results of this analytical method in Tables 6 and 7, R.S.D values were less than 5% for both between-day and between-week precisions, showing high level of agreement for the RA quantification in *I. rugosus*.

Table 6
Between-day precision results are expressed as mean (RSD %) of three replicates; RA, rosmarinic acid; SD, Standard Deviation; RSD, Relative Standard Deviation.

Sample	RA mg/kg 1st Day (w/w)	RA mg/kg 2nd Day (w/w)
1	1.56	1.62
2	1.58	1.64
3	1.68	1.59
4	1.68	1.71
5	1.70	1.68
6	1.51	1.72
Average	1.62	1.66
S.D.	0.08	0.05
R.S.D.	4.85634%	3.11859737%

Table 7
Between-week precision results are expressed as mean (RSD %) of three replicates; RA, rosmarinic acid; SD, Standard Deviation; RSD, Relative Standard Deviation

Sample	RA mg/kg 1st Week	RA mg/kg 2nd Week
1	1.68	1.72
2	1.57	1.62
3	1.65	1.82
4	1.69	1.75
5	1.75	1.68
6	1.66	1.71
Average	1.67	1.72
S.D.	0.06	0.07
R.S.D.	3.532704%	3.910585%

Robustness of the method was determined by applying different chromatographic conditions such as change in the flow rate, using different chemical brand and mobile phase proportions. As shown in the Table 8, the results of RA content in the *I. rugosus* samples do not show any significant change within the limit of SD < 0.2 and $p > 0.05$ against the changes in the chromatographic conditions.

Table 8
Robustness results expressed as (Mean ± SD < 0.2) and ($p > 0.05$) of three replicates; RA, rosmarinic acid.

Modified parameters	Levels	RA gm/kg	RT	$p > 0.05$
Mobile phase flow rate				
	0.98 mL/min	1.64 ± 0.06	6.24 ± 0.08	0.562
	1.00 mL/min	1.64 ± 0.08	6.31 ± 0.08	
	1.02 mL/min	1.63 ± 0.04	6.23 ± 0.11	
Chemical brand				
Acetonitrile	BDH	1.62 ± 0.09	6.25 ± 0.03	0.725
	Merck	1.63 ± 0.05	6.28 ± 0.02	
Mobile phase proportion				
Acetonitrile	29% (v/v)	1.61 ± 0.08	6.26 ± 0.07	0.893
	30% (v/v)	1.60 ± 0.07	6.23 ± 0.05	
	31% (v/v)	1.62 ± 0.09	6.25 ± 0.09	

These results, together with the fact that the found RA contents on each performed set of conditions present SD less than 0.2% and SD lower than 5% demonstrate that the method showed precise, selective and robust, remaining unaffected by deliberate changes on it.

The results for the quantification of rosmarinic acid in *I. rugosus* are summarized in Table 9 showing an average quantity of 1.60 + 0.14 gm/kg of RA in *I. rugosus* plant dry powder.

Table 9
Quantification values of RA in *I. rugosus* dry plant powder.

Sample No.	≥LOD	Quantity in plant dry powder (w/w) µg/mL	Quantity in plant dry powder (w/w) gm/kg
1	Yes	1556.00	1.56
2	Yes	1577.43	1.58
3	Yes	1675.16	1.68
4	Yes	1867.95	1.87
5	Yes	1700.62	1.70
6	Yes	1455.04	1.46
Average		1638.70	1.64
Standard Deviation		142.86	0.14
Amount of Rosmarinic acid in *Isodon rogusus*/kg		1638.70 ± 142.86	1.60 ± 0.14

Discussion

For the utilization and industrialization of rosmarinic acid as a botanical pesticidal product, the research on biosynthetic pathway of rosmarinic acid might contribute significantly. Incomplete and poor knowledge towards the biosynthetic pathway leads to limited biosynthetic pathway engineering. Biotechnological rosmarinic acid production has been achieved through cell cultures of *C. blumei* (Razzaque and Ellis, 1977). Suspension cultures of *S. officinalis* depicted that 36% of RA contents can be achieved (Kim et al., 2015). Molecular knowledge of the biosynthesis of the RA can help to maximize its production in *I. rugosus* through gene overexpresson studies. This information can also be utilized for RA production in microbes by engineering RA biosynthesis pathway, as described by Bulgakov et al. (2012), Jiang et al. (2016) and Ru et al. (2016). Therefore, research on rosmarinic acid biosynthetic pathway in *Isodon rugosus* will be helpful for industrial exploitation of rosmarinic acid.

In the biosynthesis of rosmarinic acid, eight genes have been involved as described in *Coleus blumei* by many researchers (Petersen et al., 1993, 1994; Kim et al., 2004; Eberle et al., 2009). In this study two key genes were targeted to amplify in *I. rugosus*. The targeted genes are working specifically in biosynthesis of RA. Other genes of the pathway are involved in many other alternate pathways as well. One of the targeted genes was rosmarinic acid synthase (RAS) and other was hydroxyphenylpyruvate reductase (HPPR). Rosmarinic acid biosynthetic pathway in *C. blumei*, has been thoroughly explained by Petersen et al. (1993), (2009) and Petersen (1997). During last years, the enzymes involved in this biosynthetic pathway have been investigated in cell cultures of many plant species belonging to Lamiaceae and Boraginaceae families (Weitzel and Petersen, 2011). The genes involved in biosynthesis of rosmarinic acid has been cloned from many species including *Melissa officinalis* (Weitzel and Petersen, 2010; 2011), *Salvia miltiorrhiza* (Huang et al., 2008; Song and Wang, 2009; Di et al., 2013), *Prunella vulgaris* (Kim et al., 2014; Ru et al., 2016) and *Lithospermum erythrorhizon* (Yamamura et al., 2001) and *Coleus blumei* (Kim et al., 2004; Berger et al., 2006). The precursors of the pathway are two amino acids, L-phenylalanine and L-tyrosine.

For HPPR cloning from *I. rugosus*, degenerate primers were designed by choosing highly conserved region by aligning sequences for HPPR from closely related plant species, *Perilla frutescens*, *Solenostemon scutellarioides*, *Salvia officinalis* and *Salvia miltiorrhiza* and with the help of these primers conserved region for HPPR from *I. rugosus* was amplified and sequence obtained was 388 bp in length. Then RACE PCR was performed to get full length sequence for HPPR from *I. rugosus*, by using gene specific primers based on the already amplified conserved region of HPPR in *I. rugosus*. RACE PCR results in amplification of 3' RACE product with sequence length of 251 bp and 5' RACE amplified product with sequence length of 502 bp. This is the first study reporting the cloning of HPPR gene from *I. rugosus*. HPPR, firstly defined by Grant (1989) belongs to the family of D-isomer specific 2-hydroxyacid dehydrogenase. HPPR responsible for the biosynthesis of rosmarinic acid and is considered first key enzyme in the biosynthetic pathway (Kim et al., 2004; Janiak et al., 2010). From suspension cultured cells of *C. blumei* in cell free extracts HPPR was characterized for the first time (Petersen and Alfermann, 1988; Hausler *et al*., 1991), afterwards purified and then sequenced (Kim et al., 2004). Its structure was solved by Janiak et al, 2010. It is active in the form of homodimer. In the biosynthetic pathway of rosmarinic acid it is involved in the reduction of 4-hydroxyphenylpyruvate with the use of NAD(P)H, which is obtained through transamination of L-tyrosine, to D-4-hydroxy phenyllactate (Petersen et al., 1993; Petersen, 1997). As indicated in the dendrogram in Fig. 6, the phylogenetic observation showed high levels of similarity of HPPR genes among the different plant species belongs to Lamiaceae family. The phylogenetic tree complements the sequence alignment results for the HPPR gene. It shows the similarity of the gene to its closely related counterparts especially to *P. frutescens*.

For cloning of RAS gene from *I. rugosus*, degenerate primers were designed by choosing highly conserved region by aligning sequences for RAS from closely related plant species, *Melissa officinalis*, *Perilla frutescens*, *Salvia miltiorrhiza* and *Solenostemon scutellarioides*. Three sets of primers were designed but no amplification of RAS from *I. rugosus* was observed or sometimes multiple bands appeared. It is possible that the RAS gene in *I. rugosus* does not share high similarities to the RAS gene in the other related plant species. This will make it difficult to amplify a target region in the RAS gene using degenerate primers designed from the RAS gene of closely related plant species. However we cannot make strong conclusions based on this. Further tests will be required to fully investigate if the RAS gene is present, absent or different. Nevertheless, the HPPR gene also plays a key role in RA biosynthesis; hence identifying the presence of this gene (HPPR) equally proves that the RA biosynthetic pathway is present in *I. rugosus*. In biosynthetic pathway, RAS transestrified D-4-hydroxyphenyllacted with 4-coumaroyl moiety obtained from 4-coumaroyl-CoA, to 4-coumaroyl-40-hydroxy phenyllactate and then by cytochrome P450 monooxygenase reactions hydroxylated to rosmarinic acid (Petersen, 1997; Petersen and Simmonds, 2003). It acts in the form of monomer, belongs to the family of BAHD acyltransferase (Berger et al., 2006).

HPPR and RAS were reported to be identified and described their involvement in biosynthesis of rosmarinic acid in *Coleus blumei* Benth (Petersen and Alfermann 1988; Hausler *et al*., 1991; Petersen, 1991; 1997; Petersen et al., 1993, 2009), *Melissa officinalis* (Weitzel and Petersen, 2011) and *Prunella vulgaris* (Kim et al., 2014; Ru et al., 2016).

In order to analyze the involvement of HPPR and RAS in the biosynthesis of rosmarinic acid, Hucherig and Petersen (2012) conducted a study in which they confirmed that both of these enzymes participate in biosynthesis of RA. For this purpose, they established the hairy root lines of *C. blumei* having constructs of HPPR or RAS RNAi overexpression and suppression. Hairy roots lines showed low RA content up to 92% reduction as compared to control when levels of HPPR or RAS mRNA were lowered. When HPPR levels were increased, RA content increased up to 176% in hairy root lines with overexpression, while overexpression of RAS resulted in reduced level of RA due to co-suppression effects.

Rosmarinic acid quantification in *I. rugosus* was done by a fast and simple RP-HPLC method, in isocratic conditions using C18 column and detection at 310 nm. System suitability tests and method validation steps provided data of acceptable quality. Results revealed that the method was selective, linear for concentration ranges between 3.12–100 µg/mL, sensitive, precise, and robust. System suitability tests assured the resolution and reproducibility of the chromatographic system satisfactory for the analysis to be performed. The system suitability test of RA indicates that all the system performance parameters (tailing factor, theoretical plate value, repeatability and system precision) are in accordance with the literature specifications (US Food and Drug Administration, 2013; Sangita et al., 2013). During selectivity test interference of no other compound was found with the retention time of RA standard and plant extract, at six different concentrations. Calibration curves showed a linear response for RA standard within the range of 3.12–100.0 ppm over a 3-day validation period. Similarly calibration curves for *I. rugosus* plant extract samples for 6 concentrations were found linear over a 3 day validation period. Calibration curve values for both standard and sample verified the analytical method suitable for RA quantification in *I. rugosus*.

Sensitivity of the analytical method was assessed by means of the LOD and LOQ values which proved that the present method has sensitivity to detect the RA in concentrations greater than 1.32 µg/ml, and quantify up to 4.41 µg/ml with adequate precision and accuracy. In previous methods, LOD and LOQ values of 0.708 and 2.361 µg/mL, and LOD and LOQ values of 7.24 and 24.13 ng/mL are reported for RA quantification in Rosemary (Wang et al.,2004; Couto et al., 2011). Precision tests were performed for the validation of method. The RSD% values < 5% for both between-day and between-week precisions, showed high level of agreement for the RA quantification in *I. rugosus*.

The robustness of the method was assessed by changing some chromatographic conditions such as flow rate, chemical brand and mobile phase proportion. All of the changes made has shown no significant change in RA contents in *I. rugosus*, as confirmed by the P values > 0.05 as shown in. These results, together with the fact that the obtained RA contents on each performed set of conditions present RSD lower than 5% demonstrate that the method is robust and remains unaffected by deliberate changes on it.

Quantity of 1.60 + 0.14gm/kg or 1638 ppm of RA in *I. rugosus* plant dry powder was calculated by this method. In the current study the quantification of RA from the *I. rugosus* was done for the first time and allowed us to conclude that it can be successfully applied on the routine quality control of rosmarinic acid analysis.

Conclusion

In this study it was confirmed that HPPR expressed in all parts of I. rugosus, including roots, stems and leaves. As, HPPR involved in the biosynthesis of rosmarinic acid is confirmed by many previous studies as stated above, this plant can be utilize as a potential source for rosmarnic acid. This is a premier study reporting expression and sequence analysis of hydroxyphenylpyruvate reductase gene responsible for synthesis of rosmarinic acid in *I. rugosus.The information about sequences obtained in the study will increase the knowledge of I. rugosus, as well as offer a reference tool for future biosynthetic genes studies associated with RA of the species.A novel HPLC method for quantification of RA in methanolic extract of I. rugosus was found selective, sensitive, precise, robust and linear over the concentration range between 3.12-100µg/mL. Quantity of 1.60+0.14gm/kg with notable sensitivity of LOD 1.32 µg/ml, and LOQ up to 4.41 µg/ml of rosmarinic acid in I. rugosus dry powder is reported in this study for the very first time.*

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SK performed the molecular analyais and AI performed the HPLC analysis. CNTT helps SK to conduct and analyzed the molecular part of the study. GS, IUH and MMS provides facilities and supervision to conduct the study.

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Figures

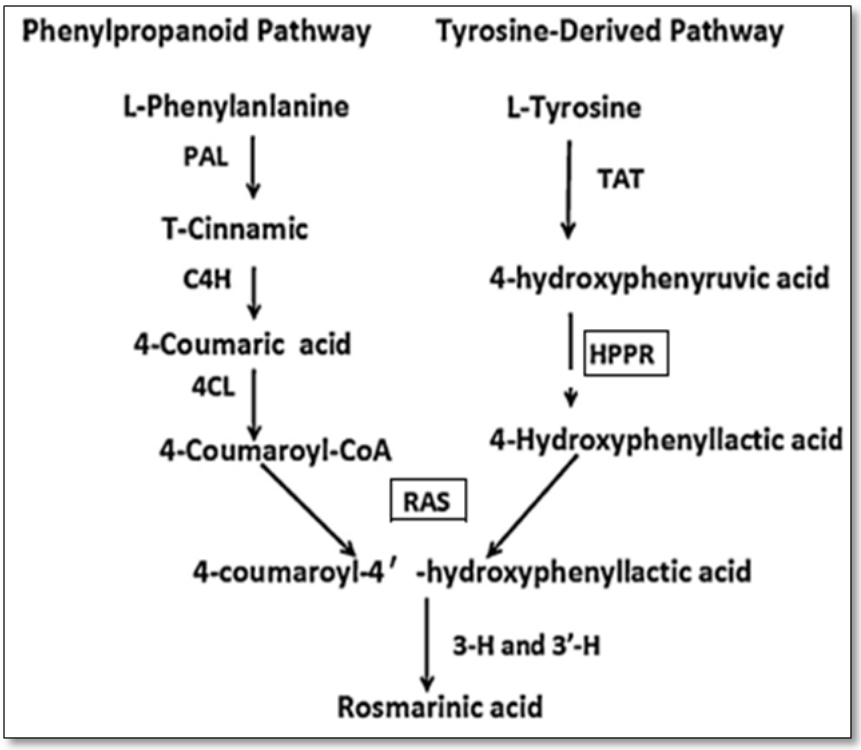

Figure 1

Biosynthetic pathway of isolated pure compound, rosmarinic acid indicating two keys genes i.e. RAS (rosmarinic acid synthase gene) and HPPR (Hydroxyphenylpyruvate reductase)

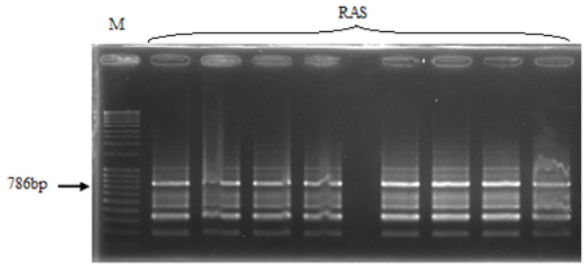

Figure 2

Multiple bands appearance for RAS, required band should be at 786 bp, M= Molecular weight marker

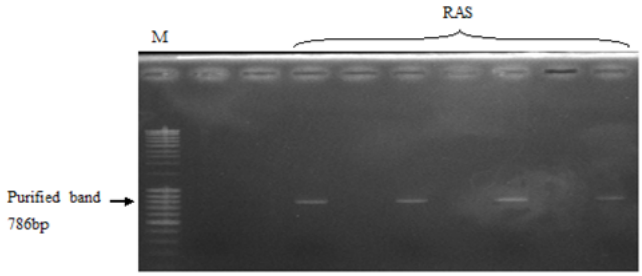

Figure 3

Purified band for RAS at 786 bp, M= Molecular weight marker

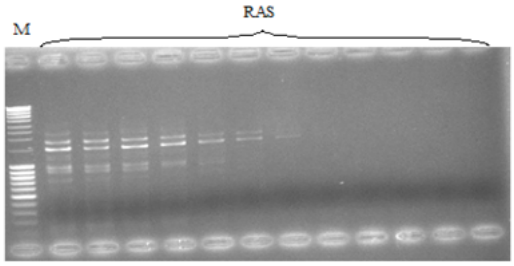

Figure 4

PCR products (multiple bands) for RAS in gradient PCR using 10 annealing temperatures between 50°C to 60°C, M= Molecular weight marker

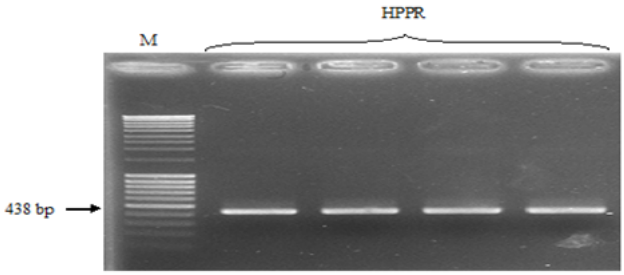

Figure 5

Purified PCR product for sHPPR at 438 bp from *I. rugosus*, M= Molecular weight marker

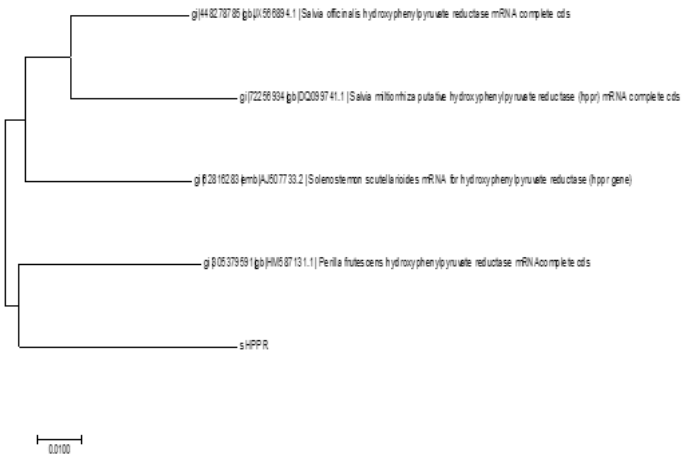

Figure 6

Phylogenetic tree for sHPPR (HPPR from *I. rugosus*) with HPPR in other plant species; *Perilla frutescens*, *Solenostemon scutellarioides*, *Salvia officinalis* and *Salvia miltiorrhiza*.

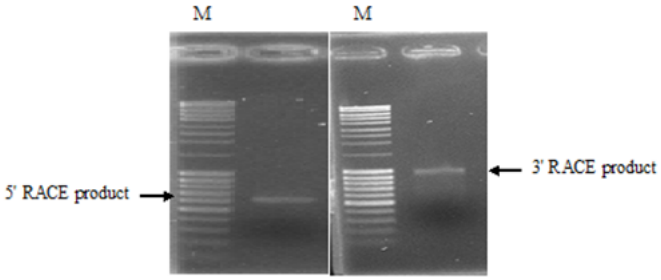

Figure 7

Purified 5' and 3' RACEPCR products for sHPPR, M= Molecular weight marker

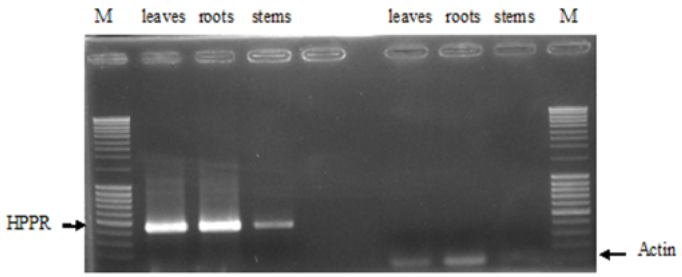

Figure 8

PCR products of HPPR and actin from *I. rugosus* leaves, roots and stems, M= Molecular weight marker

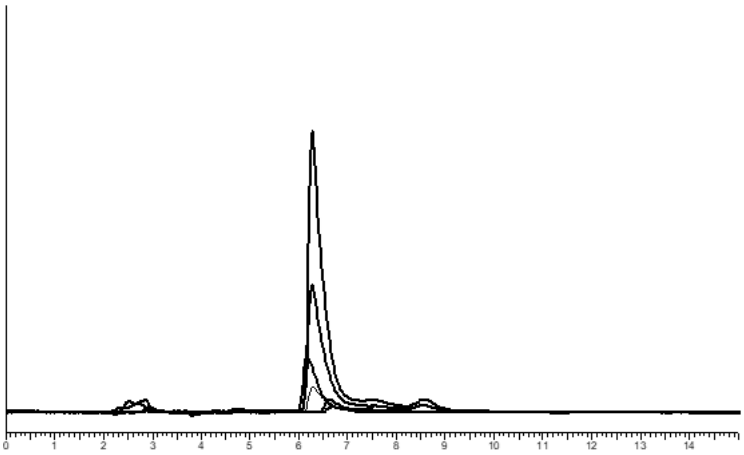

Figure 9

Typical chromatogram of rosmarinic acid standards (3.12 - 100 µg.mL^{-1}) in methanol.

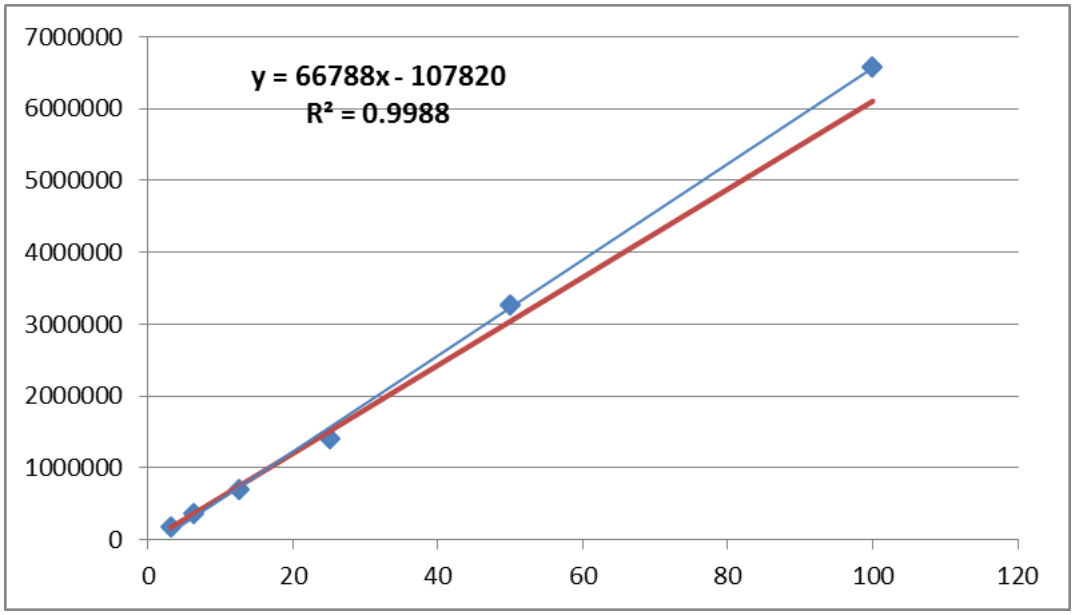

Figure 10

Calibration curve for Rosmarinic Acid (RA) Standard.

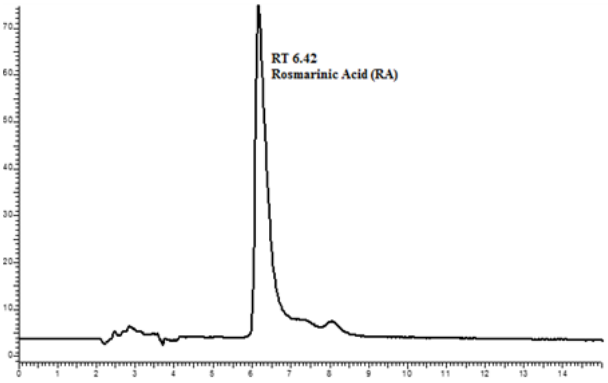

Figure 11

Typical chromatogram of rosmarinic acid standard (25 µg.mL^{-1}) in methanol.

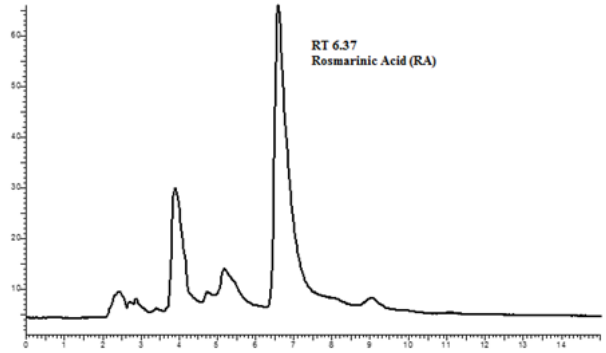

Figure 12

Typical chromatogram of Isodon rugosus plant methanolic extract indicating rosmarinic acid concentration of 28.62 µg.mL^{-1}

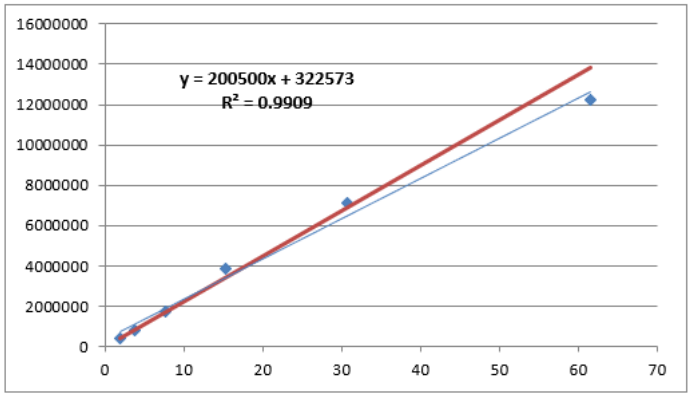

Figure 13

Calibration curve for RA in *I. rugosus* plant samples.

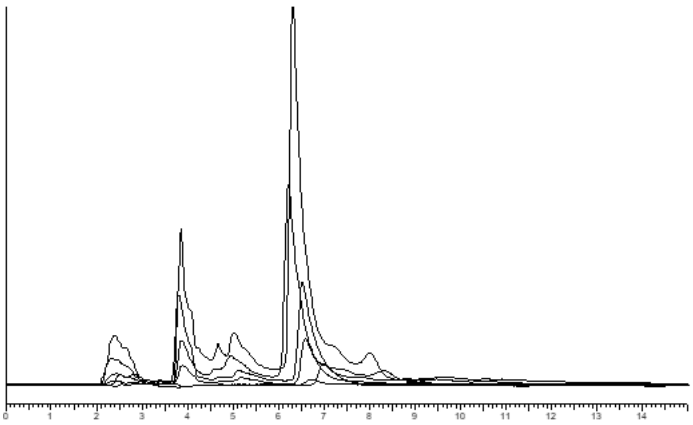

Figure 14

Typical chromatogram of RA in *I. rugosus* sample for six succeeding concentrations.